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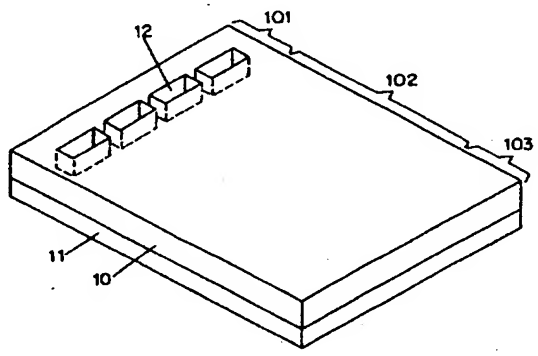
(57) Abstract

An apparatus for processing samples containing DNA to produce a sequencing fragment mixture comprises a sample processing element comprising: a thermocycling region having one or more chambers for receiving a DNA sequencing reaction mixture and forming sequencing fragments therefrom; a separation region comprising a separation matrix for separating the sequencing fragments formed in the thermocycling regions; a detection region for detection of the separated sequencing fragments; and means for regulating the temperature in the thermocycling region of the sample processing element to provide a plurality of thermal cycles, each cycle including at least a denaturation phase and an extension phase. The apparatus for processing sample can be placed in a holder which is associated with means for applying an electric field to the separation region of a sample processing apparatus placed within the holder to cause polynucleotide sequencing fragments to migrate through the separation region from the thermocycling region to the detection region; and means for detecting polynucleotide fragments within the detection region of the sample processing apparatus placed within the holder.



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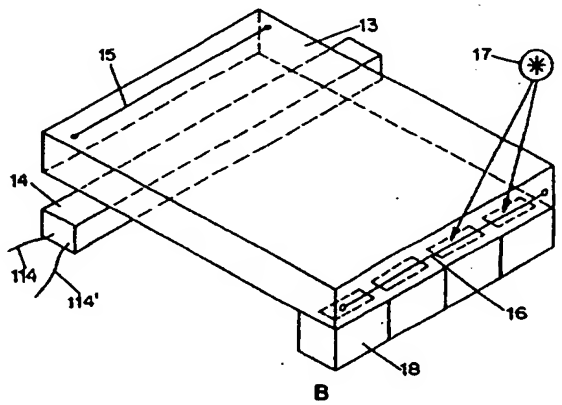

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DESCRIPTION

APPARATUS AND METHOD FOR PERFORMING SEQUENCING OF NUCLEIC ACID POLYMERS

BACKGROUND OF THE INVENTION

This application relates to apparatus for performing DNA sequencing reactions, and in particular to improved apparatus for performing sequencing reaction Protocols making use of thermally stable polymerase enzymes having enhanced capacity to incorporate chain terminating nucleotides during chain termination sequencing reactions.

DNA sequencing is generally performed using techniques based on the "chain termination" method described by Sanger et al., *Proc. Nat'l Acad. Sci.* (USA) 74 (12): 5463-5467 (1977) . Basically, in this process, DNA to be tested is isolated, rendered single stranded, and placed into four vessels. In each vessel are the necessary components to replicate the DNA strand, i.e., a template-dependant DNA polymerase, a short primer molecule complementary to a known region of the DNA to be sequenced, and the standard deoxynucleotide triphosphates (dNTP's) commonly represented by A, C, G and T, in a buffer conducive to hybridization between the primer and the DNA to be sequenced and chain extension of the hybridized primer. In addition, each vessel contains a small quantity of one type (i.e., one species) of dideoxynucleotide triphosphate (ddNTP), e.g. dideoxyadenosine triphosphate (ddA).

In each vessel, the primer hybridizes to a specific complementary site on the isolated DNA. The primers are then extended, one base at a time to form a new nucleic acid polymer complementary to the isolated pieces of DNA. When a dideoxynucleotide triphosphate is incorporated into the extending polymer, this terminates the polymer strand and prevents it from being further extended. Accordingly, in each vessel, a set of extended polymers of specific lengths are formed which are indicative of the positions of the nucleotide corresponding to the dideoxynucleotide in that vessel. These sets of polymers are then evaluated using gel electrophoresis to determine the sequence.

Improvements to the original technique described by Sanger et al. have included improvements to the enzyme used to extend the primer chain. For example, Tabor et al. have described enzymes such as T7 DNA polymerase which have increased processivity, and

increased levels of incorporation of dideoxynucleotides. (*See* US Patent No. 4,795,699 and EP-A-0 386 857, which are incorporated herein by reference). More recently, Reeve et al. have described a thermostable enzyme preparation, called ThermoSequenase™, with improved qualities for DNA sequencing. *Nature* 376: 796-797 (1995); EP-A-0 655 506, which is incorporated herein by reference. For sequencing, the ThermoSequenase™ product is used with an amplified DNA sample containing 0.5-2 µg of single stranded DNA (or 0.5 to 5 µg of double stranded DNA) into four aliquots, and combining each aliquot with the ThermoSequenase™ enzyme preparation, one dideoxynucleotide termination mixture containing one ddNTP and all four dNTP's; and one dye-labeled primer which will hybridize to the DNA to be sequenced. The mixture is placed in a thermocycler and run for 20-30 cycles of annealing, extension and denaturation to produce measurable amounts of dye-labeled extension products of varying lengths which are then evaluated by gel electrophoresis. EP-A-0 655 506 further asserts that ThermoSequenase™ and similar enzymes can be used for amplification reactions.

Each of the processes known for determining the sequence of DNA can be preceded by amplification of a selected portion of the genetic material in a sample to enrich the concentration of a region of interest relative to other DNA. For example, it is possible to amplify a selected portion of a gene using a polymerase chain reaction (PCR) as described in U.S. Patents Nos. 4,683,194, 4,683,195 and 4,683,202, which are incorporated herein by reference. This process involves the use of pairs of primers, one for each strand of the duplex DNA, that will hybridize at a site located near a region of interest in a gene. Chain extension polymerization (without a chain terminating nucleotide) is then carried out in repetitive cycles to increase the number of copies of the region of interest many times. The amplified polynucleotides are then separated from the reaction mixture and used as the starting sample for the sequencing reaction. Gelfand et al. have described a thermostable enzyme, "Taq polymerase," derived from the organism *Thermus aquaticus*, which is useful in this amplification process. (See US Patent Nos. 5,352,600 and 5,079,352 which are incorporated herein by reference).

Ruano and Kidd, *Proc. Nat'l. Acad. Sci. (USA)* 88: 2815-2819 (1991) and U.S. Patent No. 5,427,911, which are incorporated herein by reference, describe a process which they call "coupled amplification and sequencing" (CAS) for sequencing of DNA. In this process, a

sample is treated in a first reaction stage with two primers and amplified for a number of cycles to achieve 10,000 to 100,000-fold amplification. A ddNTP is then added during the exponential phase of the amplification reaction, and the reaction is processed for additional thermal cycles to produce chain-terminated sequencing fragments. The CAS process does not achieve the criteria set forth above for an ideal diagnostic assay because it requires an intermediate addition of reagents (the ddNTP reagents). This introduces and opportunity for error or contamination and increases the complexity of any apparatus which would be used for automation.

While the methods now available for DNA sequencing produce useful results, they all involve multiple steps and are carried out in multiple pieces of apparatus usually including at least a thermocycling apparatus for performing an initial amplification, an apparatus for performing a sequencing reaction, and an electrophoresis apparatus for separating the sequencing reaction products. In some cases, the detection of the sequencing reaction products is performed in real time, and the detection system is incorporated as part of the electrophoresis apparatus. In others, the detection of the sequencing fragments is performed after the separation is completed using a further piece of apparatus. While this use of multiple pieces of apparatus is reasonably well-suited for use in a research environment, where the sequence of genetic materials is being determined for the first time, it is less well-suited for use in a routine diagnostic procedure wherein the sequence of the same region of DNA is determined over and over again in multiple patients. For this latter purpose, it would be desirable to have a single apparatus which could perform the complete processing of a complex DNA sample, for example genomic or other natural abundance DNA. It is the object of the present invention to provide such an apparatus and method.

SUMMARY OF THE INVENTION

The present invention provides an apparatus and associated sample processing element for performing sequencing of a DNA containing sample, particularly a sample of genomic or other natural abundance DNA. The apparatus comprises

(a) a holder for receiving a sample processing element having a thermocycling region, a separation region and a detection region;

(b) means for regulating the temperature within the thermocycling region of a sample processing element placed within the holder;

(c) means for applying an electric field to the separation region of a sample processing element placed within the holder to cause polynucleotide fragments formed in the thermocycling region to migrate through the separation region from the thermocycling region to the detection region; and

(d) means for detecting polynucleotide fragments within the detection region of a sample processing element placed within the holder.

Sequencing is performed by loading a genomic or natural abundance DNA-containing sample; a thermostable polymerase such as ThermoSequenase™ which incorporates dideoxynucleotides into an extending nucleic acid polymer at a rate which is no less than about 0.4 times the rate of incorporation of deoxynucleotides; two primers which bind to complementary strands of a target DNA molecule at sites flanking a region of interest; a mixture of nucleotide triphosphates (A, C, G and T) and one dideoxynucleotide triphosphate into the thermocycling region of a sample processing element placed within the apparatus, and processing the combination through multiple cycles of annealing, extension and denaturation to form a mixture of sequencing fragments within the thermocycling region. An electric field is then applied to the sample processing element to cause the sequencing fragments to migrate from the thermocycling region, through the sample processing element and to the detection region. As the fragments pass through the detection region, they are detected and the output signal is analyzed to yield the sequence of the region of interest within the target sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B show an embodiment of a sample processing element and apparatus in accordance with the invention;

Fig. 2 shows a further embodiment of the apparatus of the invention;

Fig. 3 shows a section view through the thermocycling region of an embodiment of a sample processing element in accordance with the invention;

Fig. 4 shows an embodiment of an apparatus of the invention;

Fig. 5 shows an embodiment of an apparatus of the invention;

Fig. 6 shows a further embodiment of a sample processing element in accordance with the invention;

Fig. 7 shows a thermocycling and concentration chamber useful as part of a sample processing element in accordance with the invention;

Fig. 8 shows a further embodiment of the invention;

Fig. 9 shows an embodiment of a thermocycling chamber useful in the present invention;

Fig. 10 shows the positioning of a denaturing buffering in an electrophoresis gel forming part of a sample processing element of the invention;

Figs 11A and 11B show sequencing fragments patterns obtained using ThermoSequenase™ or Vent/Sequitherm; and

Fig. 12 shows a sequencing fragment pattern obtained using ThermoSequenase™.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an apparatus which helps to exploit the properties of enzymes like ThermoSequenase™, namely the ability to incorporate dideoxynucleotides into an extending polynucleotide at a rate which is no less than about 0.4 times the rate of incorporation of deoxynucleotides, to enable sequencing of a nucleic acid polymer from a sample in a single apparatus. Although the apparatus and sample processing element of the invention can be used to analyze any DNA containing sample including samples which have been previously amplified, they are particularly suited for analysis of samples containing natural abundance DNA. As used herein a "natural abundance sample" is a sample which has been treated to make DNA in the sample accessible for hybridization with oligonucleotide primers, for example by lysis, centrifugation to remove cellular debris and proteolytic digestion to expose the DNA, but which has not been subjected to a preferential purification or amplification step to increase the amount of target DNA relative to non-target DNA present in the initial sample. The term "natural abundance" does not, however, require the presence of all the DNA from the original sample. Thus, a complex sample containing just nuclear DNA, or just mitochondrial DNA or some subfraction of nuclear or mitochondrial DNA obtained by isolation from a tissue sample but not subjected to preferential amplification would be a "natural abundance" sample within the meaning of that term in the specification and claims of

this application. The term "natural abundance" would also include a DNA sample prepared by conversion, for example by reverse transcription, of a total mRNA preparation or the genome of an RNA virus to cDNA; DNA isolated from an individual bacterial colony growing on a plate or from an enriched bacterial culture; and a viral DNA preparation where substantially the entire viral genome is isolated. The term "natural abundance" does not encompass a sample in which the isolated DNA is not a complex combination of DNA molecules, and thus would not encompass, for example, a purified plasmid preparation containing only a single species of plasmid.

A first embodiment of the apparatus of the present invention comprises four basic elements:

(a) a holder for receiving a sample processing element having a thermocycling region, a separation region and a detection region;

(b) means for regulating the temperature within the thermocycling region of a sample processing element placed within the holder;

(c) means for applying an electric field to the separation region of a sample processing element placed within the holder to cause polynucleotide fragments formed in the thermocycling region to migrate through the separation region from the thermocycling region to the detection region; and

(d) means for detecting polynucleotide fragments within the detection region of a sample processing element placed within the holder.

The sample processing element of the present invention can be considered as three functional regions: a thermocycling region, a separation region and a detection region. As will be apparent from the various embodiments discussed below, these regions can be parts of integrated device, or can be separate component parts.

In general, the thermocycling region is a chamber in which the chemical reactants necessary for forming sequencing fragments directly from DNA are placed and exposed to cycles of temperature effective to promote denaturation and annealing/extension. This can be achieved by varying the temperature of the entire thermocycling region or by creating discrete temperature bands within the region.

The separation region of the sample processing element can be any type of separation matrix that is effective to separate DNA sequencing fragments on the basis of fragment size.

Thus, while the examples below refer to separation regions made from electrophoresis gels, it will be understood that other types of separation matrices, including the separation matrices described in international Patent Applications Nos. PCT/US96/09999 and PCT/US96/10110 filed June 7, 1996 and incorporated herein by reference, may also be used. The detection region of the sample processing element may be a contiguous part of the separation region, distinguishable only by the fact that detection of separated fragments occurs in this region. The detection region may also be a discrete part of the sample processing element, however, in which fragments are detected after leaving the separation region.

Figs. 1A and 1B show a first embodiment of the sample processing element and apparatus of the present invention. The sample processing element is a separation matrix such as an electrophoresis gel 10, optionally supported by a substrate 11. The electrophoresis gel 10 has three functional regions: a thermocycling region 101, a separation region 102 and a detection region 103. Within the thermocycling region 101 are a plurality of wells 12 into which the reaction mixtures for the production of sequencing fragments are placed.

A sample processing element formed from a polyacrylamide gel 10 and substrate 11 are placed within a holder 13 of an apparatus as shown in Fig. 1B. The holder 13 positions the polyacrylamide gel 10 such that the wells 12 are in alignment with a temperature regulating element, for example a Peltier heating and cooling device 14 powered via leads 114 and 114'. The holder 13 also positions the sample processing element with respect to electrodes 15 and 16 which are used to generate the electrophoretic field with the polyacrylamide gel 10, and places the detection zone 103 of the sample processing element in alignment with a light source 17 for supplying excitation suitable for excitation Of fluorescent labels on the sequencing fragments and an array of detectors 18 for detecting emission from the fluorescent labels.

Regulating the temperature of the thermal cycling region can be achieved by a variety of methods. One method, illustrated in Fig. 1B, shows the use of a Peltier device 14 for heating and cooling. These devices can maintain a temperature of 0°C to 100°C within +/- 0.5 °C. A limitation on these devices is that the speed of cooling may result in a requirement for a potentially significant period of time in which to effect the transition between the temperatures. These temperature changes can be quite large, for example from a 94°C denaturation temperature to a 45°C annealing temperature. This long time period is useable for some applications, but in others it will lead to the appearance of spurious bands and false priming.

A preferred apparatus employs a relatively large cooling sink 20 in the thermal cycling region 101 as shown in Fig. 2. The sink temperature is maintained below the minimum cycling temperature, at 4 to 35° C, preferably about 25°C. Reagents are loaded into the wells 12 within the thermocycling region of the sample processing element, where they begin to equilibrate to the temperature of the sink 20. To begin the reaction, a radiation source 21 directed to the thermocycling zone 101 is switched on. The radiation 210 may be microwave, visible light, infrared radiation or any other radiation that can be absorbed by the sample and that will not substantially damage the reactants. The radiation is applied to increase the sample temperature to the desired temperature (i.e. 94°C for denaturation) . At the end of the desired time period, the radiation intensity is changed to produce the next desired temperature in the sample. The large cooling sink 20 rapidly reduces the temperature whenever the radiation source 21 is turned off, thus providing a rapid transition for the sample. The radiation intensity therefore determines the sample temperature.

The temperature of reagents with the thermocycling region 101, should be maintained within approximately +/- 0.5°C of the desired temperature. The control of the radiation intensity therefore requires careful consideration. A variety of detectors may be used to determine the sample temperature. Temperature sensitive films using liquid crystals (Edmund Scientific Co., Barrington, NJ) can determine temperature to within +/- 0.1°C. These films could be placed beside or underneath the sample wells 12, and so provide a precise method of temperature detection. Alternatively, since the refractive index of the sample will change with temperature, detectors of refractive index may be employed. Further options include temperature sensitive dyes added directly to the sample. Detection of electrical capacitance which changes with the temperature of the solution can also be used. In all the above cases, the detectors can be linked to microprocessors which change the intensity of the radiation source to obtain the desired temperature.

The apparatus of the invention also includes means for applying an electric field to the separation region of the sample processing element. This can be in the form of solution electrodes, disposed at either end of the sample processing element. The electrophoresis gel within the sample processing element is immersed in a buffer in two wells, each of which contains an electrode which is connected to a power supply for generating the electrophoretic field. Alternatively, the electrodes can be printed on the surface of the sample processing

element. Printed electrodes can be formed from a variety of materials, including indium tin oxide (ITO) or platinum, In either case, an electric field is generated between the electrodes of sufficient magnitude to cause the sequencing fragment to migrate from through the separation region 102 where they are separated into bands based upon the size the fragment.

Once the sequencing fragments are separated by electrophoresis through the separation region 102 of the sample processing element, they are detected in the detection region 103. The type of detection system employed will depend on the type of label incorporated into the sequencing fragments. The preferred type of label will be a fluorescent label, in which case detection of sequencing fragments can be achieved using fluorescence detection means, as described for example in US Patent Application Serial No. 08/353,932, which is incorporated herein by reference. Such a detection scheme is shown generally in Fig. 1B, in which a radiation source 17 provides excitation energy to fluorophores in the sequencing fragments which are detected by an array of fluorescence detectors 18. It will be appreciated that various types of light sources producing light of an appropriate wavelength for excitation of the fluorophores may be employed, including lasers, laser diodes, and light-emitting diodes. The light may be split into individual excitation beamlets for excitation of multiple detection sites (corresponding to the lanes of the gel) within the detection region using optical fibers, diffractions gratings, a spot array generation grating or other optical components. Alternatively, a plurality of light sources, one for each detection site can be used. In addition, for multi-dye applications the apparatus may provide light of several different wavelengths to the detection site, either through the use of multiple light sources or using optical filters.

The array of detectors 18 may provide a separate detector for each detection site within the detection region 103, or one detector may be aligned to collect light from several adjacent detection site. In the latter case, the excitation light beams to the adjacent sites are suitably applied to a temporally staggered fashion so that emission from the detection sites can be distinguished.

While fluorescence labeling and detection is the preferred method of practicing the invention, other types of labeling and detection can be used as well. For example, a chromophore or a chromogenic label can be used with a photometric detection system, or a strongly chiral label could be used with a polarization detection system as described in US Patent Application Serial No. 08/387,272, which is incorporated herein by reference.

The output of the detector array is an electrical signal representing the position of one more bases in the target sequence. This signal is preferably transferred to a data processing apparatus, such as a micro or minicomputer for data alignment and base calling. Data alignment and base calling is preferably performed using the techniques described in US Patent Applications Nos. 08/497,202 and 08/670,534 which are incorporated herein by reference.

A further aspect of the present invention is the sample processing element which is placed within the apparatus of the invention. This sample processing element is, as noted above, divisible into three functional regions: a thermocycling region, a separation region and a detection region. These regions are contiguous one with the other, and are arranged so that sequencing fragments produced in the thermocycling region are electrophoresed through the separation region to form discrete ,bands of polynucleotides which are detected in the detection region.

The basic component of the sample processing element of the invention is a polyacrylamide gel 10, optionally supported by a substrate 11 or a pair of substrates. In the embodiment shown in Fig.1A, the thermocycling region 101 is different from the separation and detection regions 102 and 103 (which are structurally indistinguishable from one another) by virtue of the wells 12 formed in the polyacrylamide gel to receive the sequencing reaction mixture. The wells need to hold this reaction mixture with sufficient integrity for the duration of the thermal cycling reaction. This may take up to 2 hours in some cases, although preferably it would be completed in under 30 minutes.

If a polyacrylamide gel slot is not a satisfactory reaction chamber, a cycling chamber insert, 30, may be employed, as in Fig. 3. This glass or plastic chamber insert 30 acts as liner to prevent reactant from dissolving into the buffer of the gel 32 surrounding the chamber 12. Each insert has walls but no bottom, with the substrate 31 serving as a floor. The outside of the chamber insert 30 may be in direct contact with the gel 32, if, for example, the insert 30 is put in place before the polyacrylamide gel is cast.

The chamber insert 30 receives the sample and keeps it concentrated. To prevent evaporation during the temperature cycling phase, the reactants may be layered with an oil. Alternatively, the chambers may have individual caps which are heated to keep water from condensing on the top of the chamber. The chamber insert 30 may be disposable. If it does

not have an internal valve or removable wall to release the sample after thermal cycling it can simply be removed from the gel prior to electrophoresis. A chamber insert material that dissolves after the reactions are completed could also be used. In the end, the reaction products would be conveniently situated in the wells, ready for electrophoretic analysis.

An alternative method to release reaction products after the reaction is to use a viscosity trap. This method uses a wax, oil or glycerol which acts as a solid barrier to diffusion at a cool temperature, but which will allow migration of sample when warmed or melted. By careful positioning of cooling devices, a very small amount of barrier material can be kept sufficiently cool to prevent the heated reaction products from leaking from the thermocycling region. Upon completion of the reaction, the temperature of the trap is raised to allow diffusion of the reaction products out of the thermocycling region.

Figs. 4 and 5 illustrate alternative designs for the thermocycling region of the sample processing element where the apparatus provides fixed temperature heat zones are used and the DNA sample migrate from one temperature site to the next, in sequence. The migration may be induced by electrophoresis, a thermal capillary pump (see Burns et al. 1991. Microfabricated structures for integrated DNA analysis. *Proc. Nat'l Acad. Sci. (USA)* 93: 5556- 5561), or other methods.

In Fig. 4, three separate temperature regions for denaturing, extension and annealing are established by temperature regulating elements 400, 401 and 402. These temperature regulating elements may be Peltier devices, heat exchangers, or combinations of heat sinks and radiant heaters as disclosed above. A series of reversible electrodes 40, 41, 42 and 43 are employed to move DNA back and forth between the temperature regions within a buffer reservoir. For example, a sample and accompanying reaction mixture may be initially be deposited in denaturation region D and treated for an initial denaturation time at denaturation temperature. Electrodes 40 and 43 (or 15 and 43) are then activated to cause the DNA in the sample to migrate from the denaturation region D to the annealing region A, after which time the electric field is either turned off or electrodes 42 and 43 are turned on with oscillating polarity to maintain it within the region. The DNA is allowed to rest in the annealing region A for a period of time corresponding to the desired annealing time, after which time electrodes 41 and 43 are activated to cause the annealed DNA to migrate to the extension region E and then turned off. After the desired period of time at the extension temperature, electrodes 40

and 42 are activated to cause the DNA to migrate back to the denaturation region D. This cycle of activating and deactivating electrode pairs is repeated for as many cycles as is necessary to produce a detectable amount of sequencing fragments. Then electrodes 15 and 16 are activated to cause the sequencing fragments to migrate through the separation region 102, to the detection region 103.

As an alternative to the use of electric fields to move DNA from one temperature region to another, DNA can be bound to magnetic beads, such as Dynal beads, and moved from one temperature region to the next by an electromagnet. Thus, a denatured DNA-containing sample is loaded in an annealing region, and allowed to hybridize with primers. An electromagnet is then turned on to move the DNA to the extension region. Depending on the distances involved, the strength of the magnetic field and the weight of the beads, the electromagnet may be located under the extension region, or may be a "moving" magnet which starts at the annealing region and moves to the extension region. "Moving" in this case can refer to physical movement of an electromagnet from one region to the next, or can be simulated through the use of several magnets which are activated in sequence to create the same effect.

Once the DNA is in the extension region, the electro- magnet may be switched off or maintained, at a low field strength to limit diffusion of the DNA. At this site the hybridized primers and template are exposed to all reagents, including enzymes, that are needed for primer extension/chain termination. The enzymes may be linked to a solid support and fixed at the extension site to prevent diffusion in the sample processing element. After sufficient time in the extension region, the DNA is magnetically transported to the denaturing region to separate the DNA strands. After sufficient time to complete denaturation, the separated strands are magnetically transported back to the annealing region. A fresh supply of some reagents may be required to allow for continued reactions. These reagents may be supplied by a continuous drip of fresh reagents.

It is noted that only the DNA needs to be treated to the temperature cycling, not the other reaction components. Thus, reaction components, particularly enzymes can be immobilized within the temperature region where they are needed or added on an as needed basis to the region where they are consumed.

Fig. 5 shows a variation of the apparatus shown in Fig. 4 in which one circular array of heat pads are disposed within the thermocycling region 101 for each sample. Each heating zone D (denaturation), A (annealing) and E (extension) is separately heated and maintained within +/- 0.5°C. Aqueous buffer covers the entire unit. Again, some reagents maybe immobilized or added to specific regions during the thermocycling. The reaction mixture is loaded and contained over the heat pads in the thermocycling zone 101, and DNA is drawn towards the denaturation zone D where the temperature is maintained at the denaturation temperature (i.e., 94°C) by activation of electrodes 501/502 and 505/503. After the desired amount of time, electrodes 502 and 503, are activated to draw the sample to the annealing region A. After annealing, electrodes 504 and 505, are activated to draw the DNA to the extension phase temperature region E. Finally, the cycle is completed by activating electrodes 506 and 501, to draw the DNA to the denaturing region D of the device. After sufficient temperature cycles, the sample is denatured one last time, then separated through the separation matrix upon the activation of electrodes 15 and 16.

In the embodiments shown in Figs. 4 and 5, it is only necessary that polymerase enzyme by present in the extension region E. Thus, a non-thermostable polymerase enzyme can be used if it can be successfully contained within the extension region E. This can be accomplished by immobilization of the enzyme in the extension region or by placing semipermeable membranes which restrict the passage of polymerase enzyme but not the relatively smaller DNA molecules around the extension region E.

Figs. 6 and 7 shows a further embodiment of the sample processing element of the invention in which the thermocycling region is a discrete thermocycling and concentration manifold 60 which is separable from rather than integral with the gel portion of the sample processing element. The manifold 60 is made up of an array of individual thermocycling and sample concentrating chambers 70, a single one of which is shown in Fig. 7.

The manifold 60 has a temperature regulating element 61 disposed in contact therewith for regulating the temperature of materials within the thermocycling and sample concentrating chambers 70. The manifold 60 fits directly onto the top of an electrophoresis gel, for example a Visible Genetics Inc. MicroCel™ Cassette, (50 micron thick electrophoresis slab gel) of the type described in US Patent Application No. 08/332,557 and International Patent Publication No. W096/13717, which are incorporated herein by reference. This gel has a top substrate 61

and a bottom substrate 62 surrounding a very thin gel 63. The top substrate 61 has a free beveled edge 64 which receives the manifold 60.

The manifold 60 is suitably made of a thermally conductive material to facilitate temperature regulation. The manifold may be disposable, or it may include disposable inserts.

A sequencing reaction mixture is loaded into each chamber 201 of the manifold 60, and the temperature is then cycled as required to produce sequencing fragments. The amplified DNA in the sample is then electrophoretically concentrated and loaded onto the gel 63.

As shown in Fig. 7, each sample concentrating and loading chamber consists of a large rectangular channel 201 which functions as the thermocycling chamber attached at right angles to a second smaller rectangular channel 202. The upper face of the large rectangular channel 201 is open, and receives a volume (for example 100 nL of unconcentrated sample containing a DNA mixture to be separated. The lower face of the smaller rectangular channel 202 is also open and releases the concentrated sample (approx 1 pL) into one of the functional channels of the DNA sequencing gel 63. There is an unrestricted passageway between the large and small channels, to allow sample to flow between them, at a time after a first concentration step and before a second concentration step.

In the first concentration step, sample loaded into the top of the large channel 201 is electrophoresed using a field generated between electrodes 205a and 205b. The DNA is collected on a semipermeable membrane 204a which has a molecular weight cutoff low enough to prevent passage of the DNA but which permits passage of the solvent from the sample, thereby effecting a first concentration of the sample on the semi-permeable membrane 204a. Next, a second set of electrodes 206a and 206b are turned on to generate cause the concentrated sample to migrate in a direction perpendicular to the original migration from the semipermeable membrane 204a into the small channel 202. A second semipermeable membrane 204b retains sample within the small channel 202 while permitting passage of solvent. Finally, a third electrode set 207a and 207b is used to electrophorese the doubly-concentrated sample from the small channel 202 into one of the DNA sequencing gel 63. In a variation of this device, a valve can be used in lieu of the electric fields to dispense the fragments onto the gel, but this device does not provide the reduction in sample volume of the device shown in Fig. 7.

Fig. 8 illustrates an alternative embodiment where the manifold 60 is thermal cycled separately from the gel cassette in a separate heating/cooling block 802, and then clipped onto the gel cassette 800 immediately prior to loading into the electrophoresis gel, 63. This design prevents the cassette itself from being exposed to thermal cycling conditions, which may prove detrimental to the effectiveness of the sample processing element.

Fig. 9 shows an alternative design for a thermocycling chamber which can be used in placo of or in combination with the loading/concentration manifold of Fig. 7. A capillary tube 91 is filled partway with a viscous liquid 92a such as 50% glycerol or 50% sucrose in buffer, then with the PCR solution 93 containing primers, buffer, dNTPs, genomic DNA substrate, ddNTPs and ThermoSequenase™ enzyme, and then again with further buffered viscous liquid, 92b. The diameter of the capillary is small enough that surface tension holds the different liquid layers without mixing. The two viscous layers, when brought to a low temperature, will act as a viscosity trap 8l, to confine the PCR to a small volume. The capillary is placed snugly within a channel in a thermal cycling system, 94, such as a block or fluid based heating/ cooling system. The ends of the capillary are inserted into buffer chambers 95 to.prevent evaporation. The thermocycling system is designed to maintain a temperature of approximately 0°C at the two viscosity traps, 92a and 92b, and also to provide temperature cycling (e.g. 94-55-72°C) at the central PCR solution, 93.

After thermal cycling, the capillary is removed from the thermal cycling system and the contents loaded onto a separation matrix. For example, the capillary can be inserted through a hole or notch in the top substrate of an electrophoresis gel holder, or can be expelled into conventional sample loading slots of an electrophoresis gel, or can be expelled into a chamber of a loader/concentrator of the type shown in Fig. 7. To expel the sample, the viscosity trap is first opened by warming the capillary. DNA can be moved out of the capillary and onto the separation matrix using an electric field, or (particularly in the case where the loader is used) can be simply expel led along with the trap components using mild air pressure.

In each of the Fig. 6-8 designs and in a gel loaded from the thermocycler chamber of Fig. 9, the reaction mixture may optionally be drawn through a denaturing "loading buffer" just prior to its entrance in the gel, as illustrated in Fig. 10. The entrance to gel 901, between the substrates 902, is layered with a thin wash of denaturing/stop buffer (50-100 mM formamide, plus dyes, etc), 903. The lower end of the thermocycling and concentrating

chamber 60 is placed in the buffer 903 leaving a short gap for sample to traverse between the device and the gel, 901. Electrodes 904 and 905 which are printed on the inside face of the substrate 902 can be switched on to draw the sample through the buffer layer.

The detection region of the sample processing element of the invention may simply be a continuous portion of the separation region, distinguished only by its function. If desired, however, the detection region can have structure specifically adapted to facilitate detection of the separated sequencing fragments as they pass through the detection region. Thus, for example, as disclosed in U.S. Patent Application No. 08/571,297, which is incorporated herein by reference, one substrate can be made with a thin region localized in the detection region to create a window for monitoring the detection region with decreased interference from the substrate. Such substrates can be formed by molding a contiguous substrate into the desired shape, or by affixing blocks of thicker materials onto a continuous thin substrate.: In the latter case, the blocks 71 of thicker material may also be formed from absorbing, non-fluorescing materials to further reduce background fluorescence.

The apparatus and sample processing element of the invention are utilized in the method of the invention to sequence DNA, for example the DNA found in natural abundance DNA samples. In accordance with this method, a thermostable polymerase enzyme which incorporates dideoxynucleotides into an extending polynucleotide at a rate which is no less than about 0.4 times the rate of incorporation of deoxynucleotides, a natural abundance DNA-containing sample, two primers flanking the region of DNA to be sequenced, each primer binding to a different strand of duplex DNA, and other reagents for performing an enzyme-catalyzed primer extension reaction are combined in the thermocycling region of a sample processing element. The sample processing element is then placed in the apparatus of the invention and processed through sufficient cycles to produce a detectable amount of sequencing fragments. An electric field is then generated to cause the sequencing fragments to migrate through the separation region of the sample processing element, arid thereby be separate into discrete bands on the basis of the size of the fragments. These bands are then detected as they pass through the detection region.

A key factor in successfully performing the method of the invention is the utilization of ThermoSequenase™ or a comparable enzyme as the thermostable polymerase in the reaction mixture. Such an enzyme is characterized by a high affinity for incorporating dideoxynucleo-

tides into the extending nucleotide chain. Thus, for example, ThermoSequenase™ is known to favor the incorporation of dideoxynucleotides. In general, for purposes of the present invention, the polymerase used should be one which incorporates dideoxynucleotides into an extending nucleic acid polymer at a rate which is no less than about 0.4 times the rate of incorporation of deoxynucleotides.

Figs. 11A, 11B and 12 illustrate the importance of this characteristic of the polymerase enzyme employed. Figs. 11A and 12 shows a sequencing data trace for an actual patient sample of genomic DNA which was obtained using ThermoSequenase™ and primers effective to amplify exon 2 of the Von Hippel-Lindau gene in a process according to the invention. Large, well-defined peaks corresponding to the termination fragments were obtained which made sequence evaluation of the sample very straight- forward. In addition, the peaks for homozygous peaks are all approximately the same size, and are readily distinguishable from peaks for heterozygous locations. This result was obtained performing the test in a single reaction vessel, with a single unaugmented reaction mixture, in a total of 45 thermal cycles. Comparable results could be obtained using fewer reasctions cycles, for example 35 cycles.

In contrast, Fig. 11B shows the trace obtained when a combination of Vent and Sequitherm™ was used instead of ThermoSequenase™ for a total of 45 cycles. In this trace, the peaks for the termination fragments are much smaller and less well defined. Furthermore, the peaks are quite variable in height and did not permit identification of heterozygous peaks based on peak height. Performing the same experiment using Taq polymerase resulted in a data trace that contained no usable peaks.

In actual practice, it has been found that useful results are obtained with ThermoSequenase™ when the reaction is run for 35 to 45 cycles, using a dideoxy:deoxy mole ratio of 1:100 to 1:300. Thus, in general it can be expected that mole ratios of 1:50 to 1:500 will yield acceptable results. Specific optimum levels for other enzymes found to have the appropriate affinity of incorporating dideoxynucleotides can be identified by routine optimization using the ThermoSequenase™ values as a starting point.

In the method of the present invention, the two primers used directly produce the sequencing fragments which are analyzed to determine the sequence of the DNA in the sample. Accordingly, at least one of the primers is advantageously labeled with a detectable label such as a radiolabel, a fluorophore, a chromophore, a fluorogenic or chromogenic label,

or any other label which can facilitate the detection of the sequencing fragments produced in the reaction. Some full length product (the product spanning from one primer to the other) will also be produced and will be detected during sequencing and may be a substantial band relative to any of the individual truncation products. To avoid losing information due to the size of this band, it may be advantageous to use relatively long primers, for example a 20-25 mer such that the difference in length between the full length product and the longest possible truncation product will be 21 to 26 bases.

It may also be advantageous to label both primers used in the method of the invention. For example, the second primer can be labeled with a second detectable label, preferably different in characteristics from the first label. For example, the primers can be labeled with two different fluorophores as in the process described by Wiemann et al., "Simultaneous On-Line DNA Sequencing on Both Stands with Two Fluorescent Dyes," Anal, Biochem 224-117-121 (1995). Analysis of the fragments labeled with the two different labels can be accomplished by loading aliquots of the reaction mixture onto two different electrophoresis lanes which are evaluated for different label types or by loading the product mixture onto one lane in a multi-dye sequencer which has the ability to evaluate several labels in a single instrument.

One of the important characteristics of the present invention is the fact that it permits conversion of natural abundance DNA to a sequencing product mixture in a single set of thermocycling reactions without modification of or addition to the reagents present in the reaction mixture. Natural abundance DNA can be prepared from blood or tissue samples by any of a number of techniques, including salt precipitation or standard SDS-proteinase K-phenol extraction. Natural abundance DNA can also be prepared using kits, for example the Gentra Pure Gene DNA Isolation Kit.

CLAIMS

1. An apparatus for processing samples containing DNA to produce a sequencing fragment mixture comprising

(a) a sample processing element comprising:

a thermocycling region have one or more chambers for receiving a DNA sequencing reaction mixture and forming sequencing fragments therefrom;

a separation region comprising a separation matrix for separating the sequencing fragments formed in the thermocycling regions;

a detection region for detection of the separated sequencing fragments; and

(b) means for regulating the temperature in the thermocycling region of the sample processing element to provide a plurality of thermal cycles, each cycle including at least a denaturation phase and an extension phase.

2. The apparatus according to claim 1, wherein the means for regulating the temperature is a Peltier device disposed in thermal contact with the thermocycling region of the sample processing element.

3. The apparatus according to claim 1, wherein the means for regulating the temperature comprises a heat sink maintained at a temperature below the desired temperature of the thermocycling region and a source of radiant heat.

4. The apparatus according to claim 1, wherein the means for regulating the temperature comprises at least three temperature regulating elements, a first temperature regulating element for maintaining a first portion of the thermocycling region at a denaturation temperature; a second temperature regulating element for maintaining a second portion of the thermocycling region at an annealing temperature, and a third temperature regulating element for maintaining a third portion of the thermocycling region at an extension temperature.

5. An apparatus for sequencing DNA in a sample comprising

a holder for receiving sample processing apparatus for processing samples containing DNA to produce a sequencing fragment mixture according to any of claims 1 to 4;

means for applying an electric field to the separation region of a sample processing apparatus placed within the holder to cause polynucleotide sequencing fragments to migrate through the separation region from the thermocycling region to the detection region; and

means for detecting polynucleotide fragments within the detection region of the sample processing apparatus placed within the holder.

6. The apparatus according to claim 5, wherein the means for detecting polynucleotide fragments within the detection region comprises a source of radiation for exciting a fluorescent label on the sequencing fragments and a detector for detecting fluorescent emission from the sequencing fragments.

7. The apparatus according to claim 6, wherein the means for detecting polynucleotide sequencing fragments within the detection region comprises a plurality of detectors.

8. The apparatus according to claim 6, wherein the means for detecting polynucleotide sequencing fragments comprises means for splitting radiation from the excitation source into a plurality of excitation beamlets.

9. The apparatus according to claim 8, wherein the means for splitting radiation from the excitation source into a plurality of excitation beamlets is a spot array generation grating.

10. The apparatus according to any of claims 5-9, wherein the means for applying an electric field is a pair of solution electrodes.

11. The apparatus according to any of claims 1-11, wherein the separation region comprises a polyacrylamide electrophoresis gel, and wherein the chamber is a well formed in the gel near a first end thereof.

12. The apparatus according to claim 11, further comprising a removable liner disposed within the well for separating the DNA sequencing reaction mixture from the gel during the formation of the sequencing fragments.

13. The apparatus according to any of claims 1 to 10, wherein the thermocycling region comprises a manifold formed from a plurality of chambers arranged in a line, each of said chambers in the manifold having a top opening for receiving the sequencing reaction mixture and a bottom opening for transferring sequencing reaction fragments formed in the separation region.

14. The apparatus according to claim 13, wherein each chamber of the manifold has connected thereto a concentrating device for concentrating the sequencing fragments formed therein prior to transferring them to the separation region.

15. The apparatus according to claim 13 or 14, wherein the manifold is separable from the separation region.

16. A method for sequencing a selected region within DNA molecules in a natural abundance sample comprising the steps of

(a) combining the sample with a thermostable polymerase enzyme which incorporates dideoxynucleotides into an extending polynucleotide at a rate which is no less than about 0.4 times the rate of incorporation of deoxynucleotides, two primers flanking the selected region of DNA to be sequences, each primer binding to a different strand of duplex DNA, nucleotide triphosphate feedstocks and a type of chain terminating nucleotide triphosphate to form a sequencing reaction mixture;

(b) placing the sequencing reaction mixture in an apparatus according to any of claim 1-15;

(c) processing the sequencing reaction mixture through a plurality of thermal cycles, each cycle including at elast a denaturation phase and an extension phase to form sequencing fragments;

(d) separating the sequencing fragments; and

(e) detecting the separated sequencing fragments.

17. A method for evaluating the sequence of a target polynucleotide in a sample comprising the steps of:

(a) loading the sample and reagents for forming chain-temrinated sequencing fragments into at least one chamber of a manifold formed from a plurality of chambers arranged in a line, each of said chambers in the manifold having a top opening for receiving the sequencing reaction mixture and a bottom opening for transferring sequencing fragments formed therein to a separation matrix;

(b) exposing the sample and the reagents in the chamber to a plurality of thermal cycles, each cycle including at least a denaturation pahse and an extension [hase to form seuqneicng fragments;

(c) transferring the sequencing fragment through the bottom opening to a separation matrix;

(d) separating the sequencing fragments in the separation matrix to form a plurality of discrete bands, each band reflecting one size of sequencing fragment formed;

(e) detecting the discrete bands, wherein the size of the sequencing fragments provides information concerning the sequence of the target polynucleotide.

18. The method according to claim 17, wherein the manifold is attached to the separation matrix during thermal cycling.

19. The method according to claim 17, wherein the manifold is attached to the separation matrix after thermal cycling is complete.

20. The method according to any of claims 17-19, wherein each chamber of the manifold has connected thereto a concentrating device for concentrating the sequencing fragments formed therein prior to transferring them to the separation region.

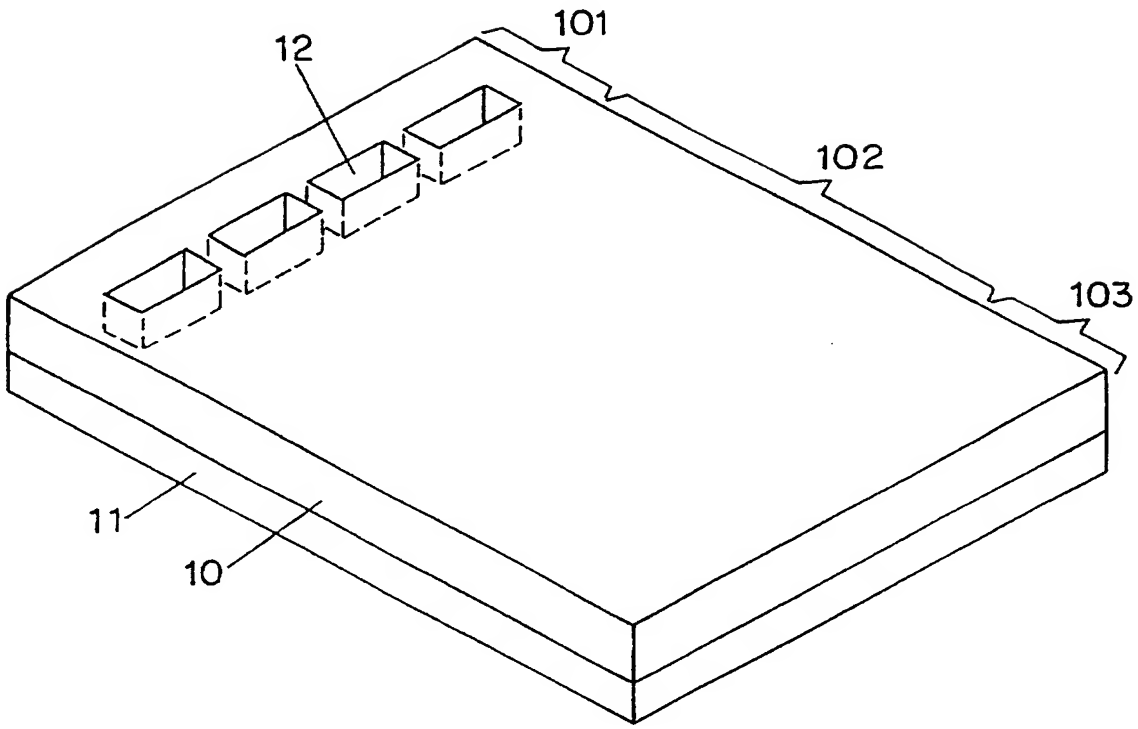

FIG. 1A

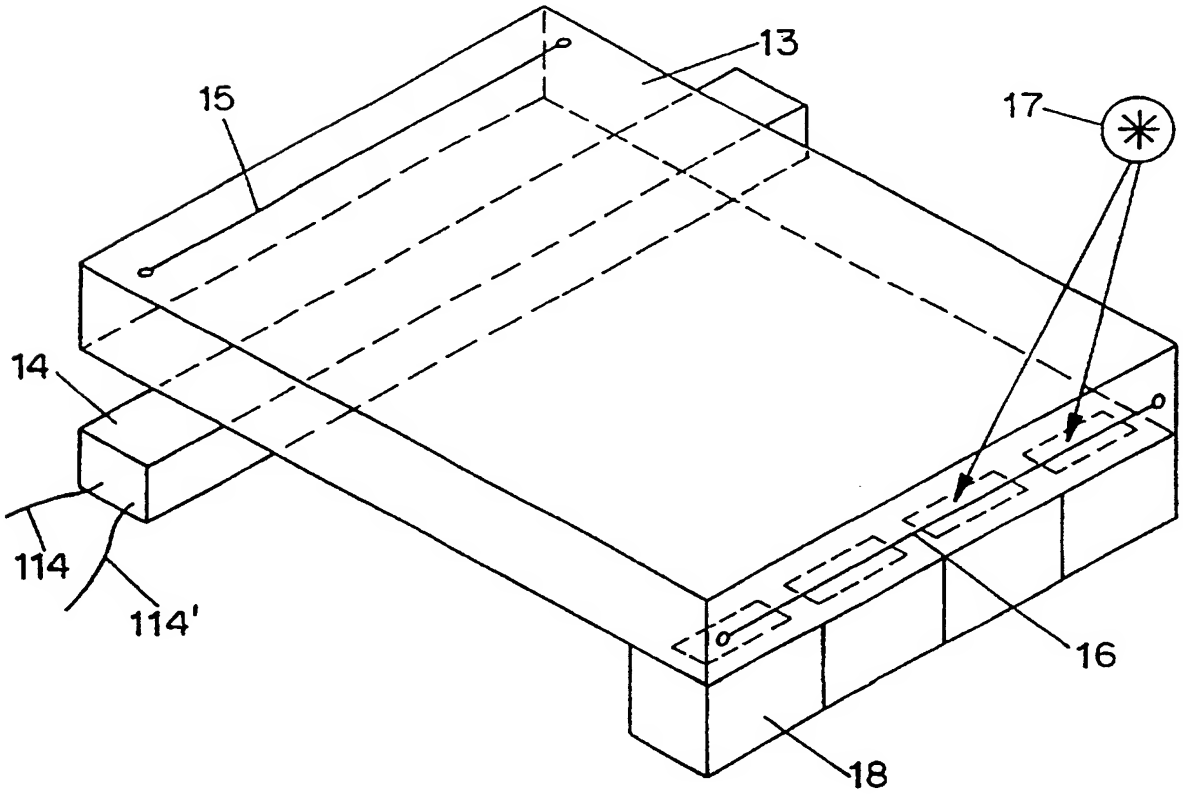

FIG. 1B

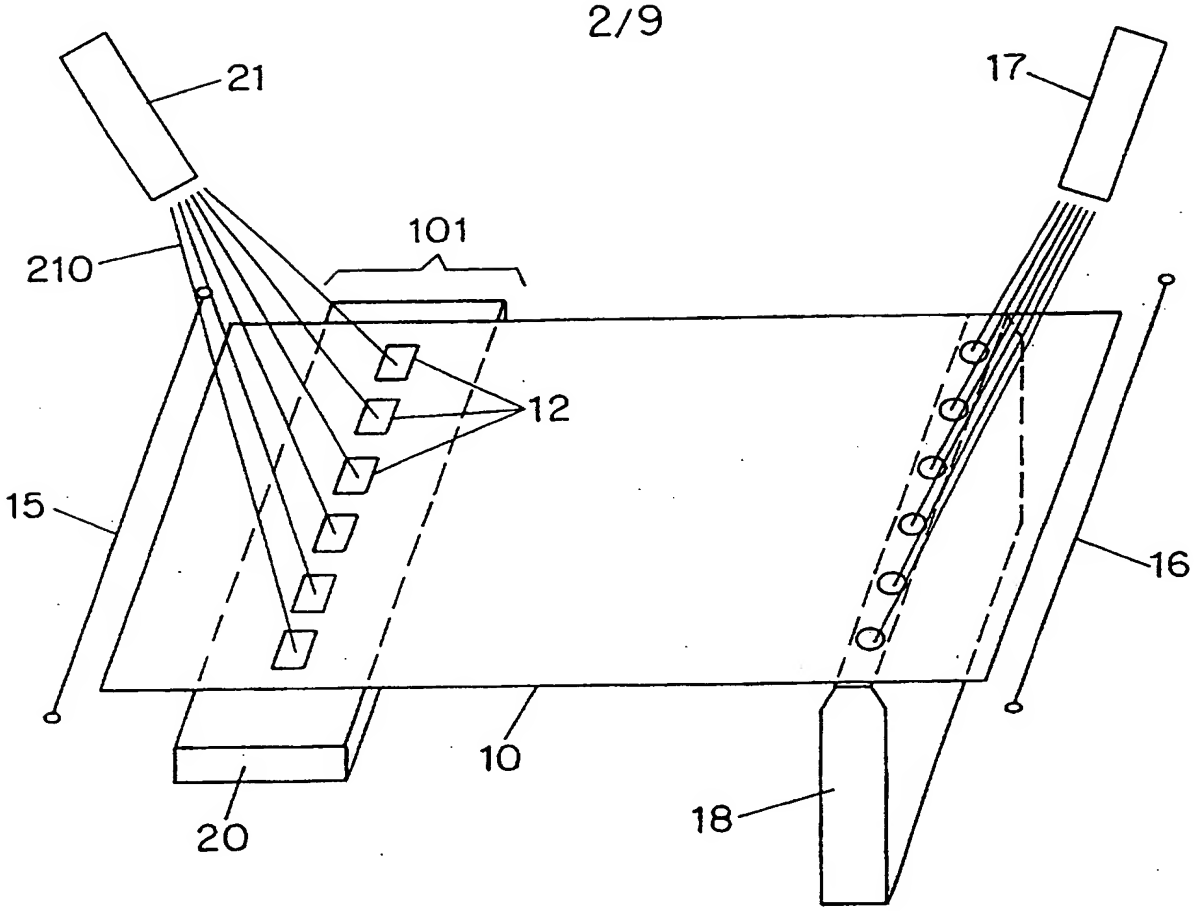

FIG. 2

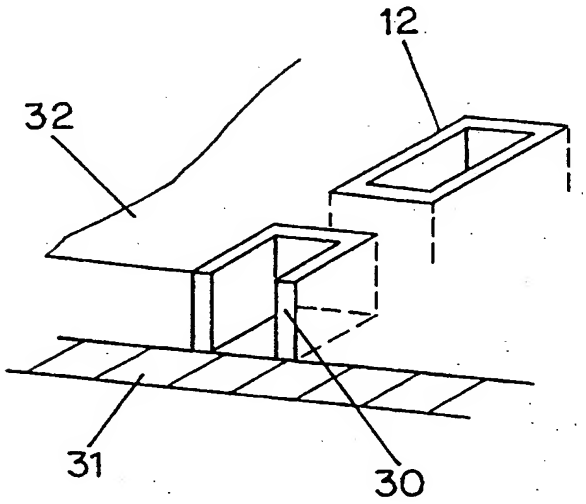

FIG. 3

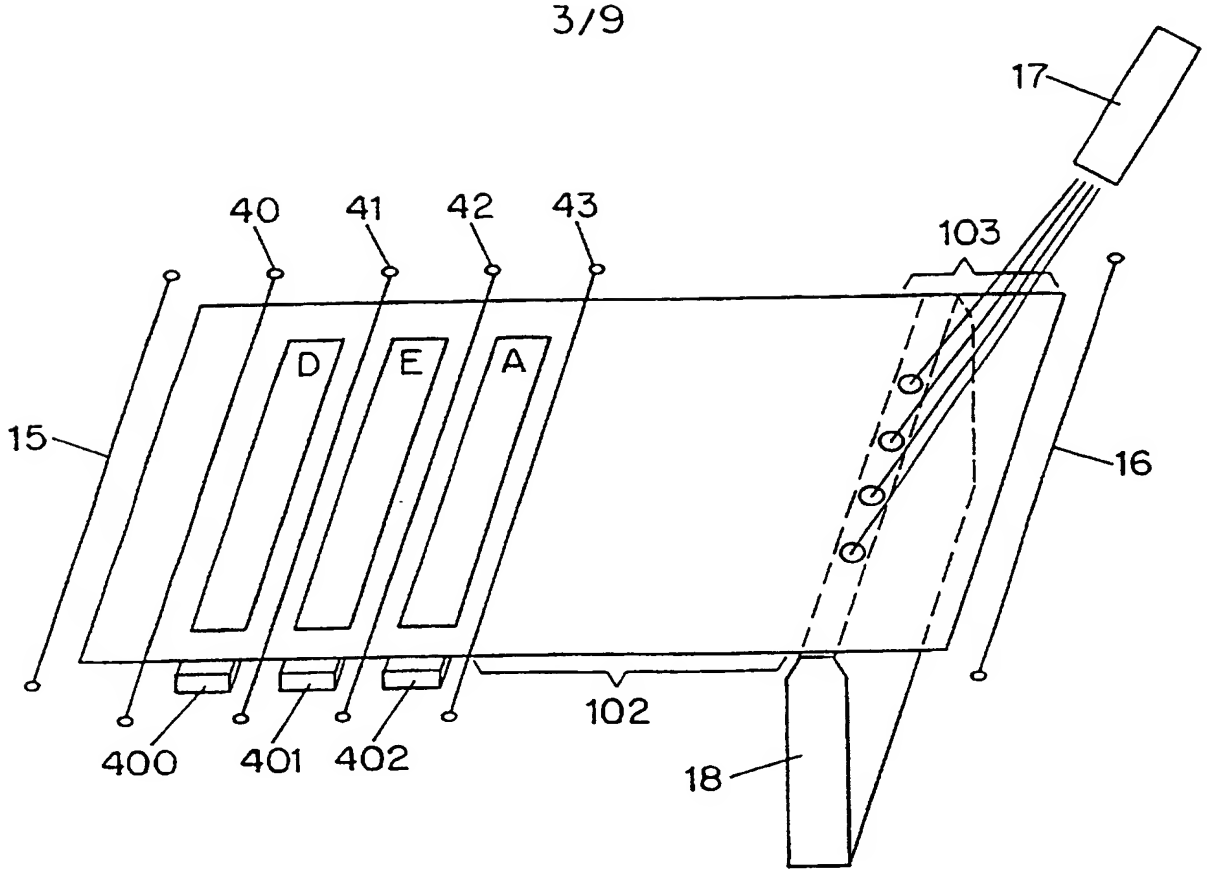

FIG. 4

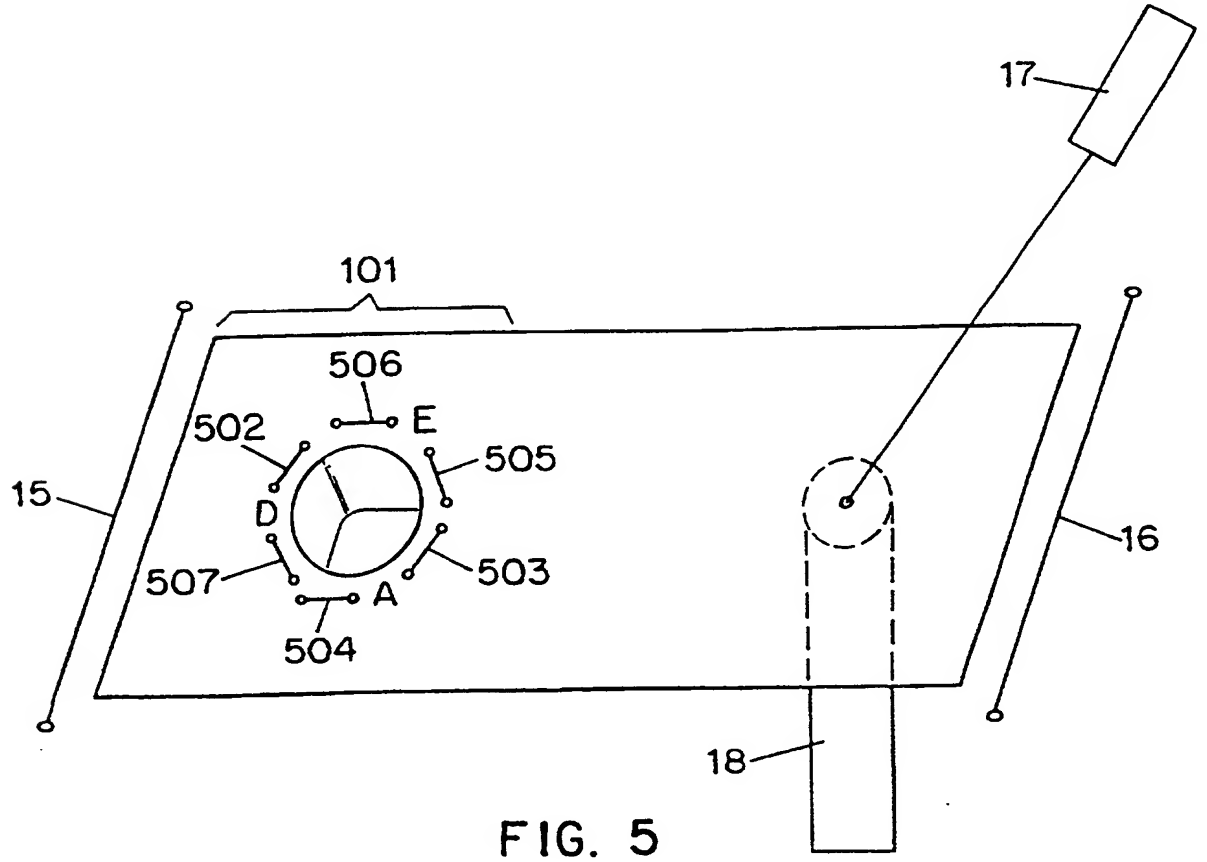

FIG. 5

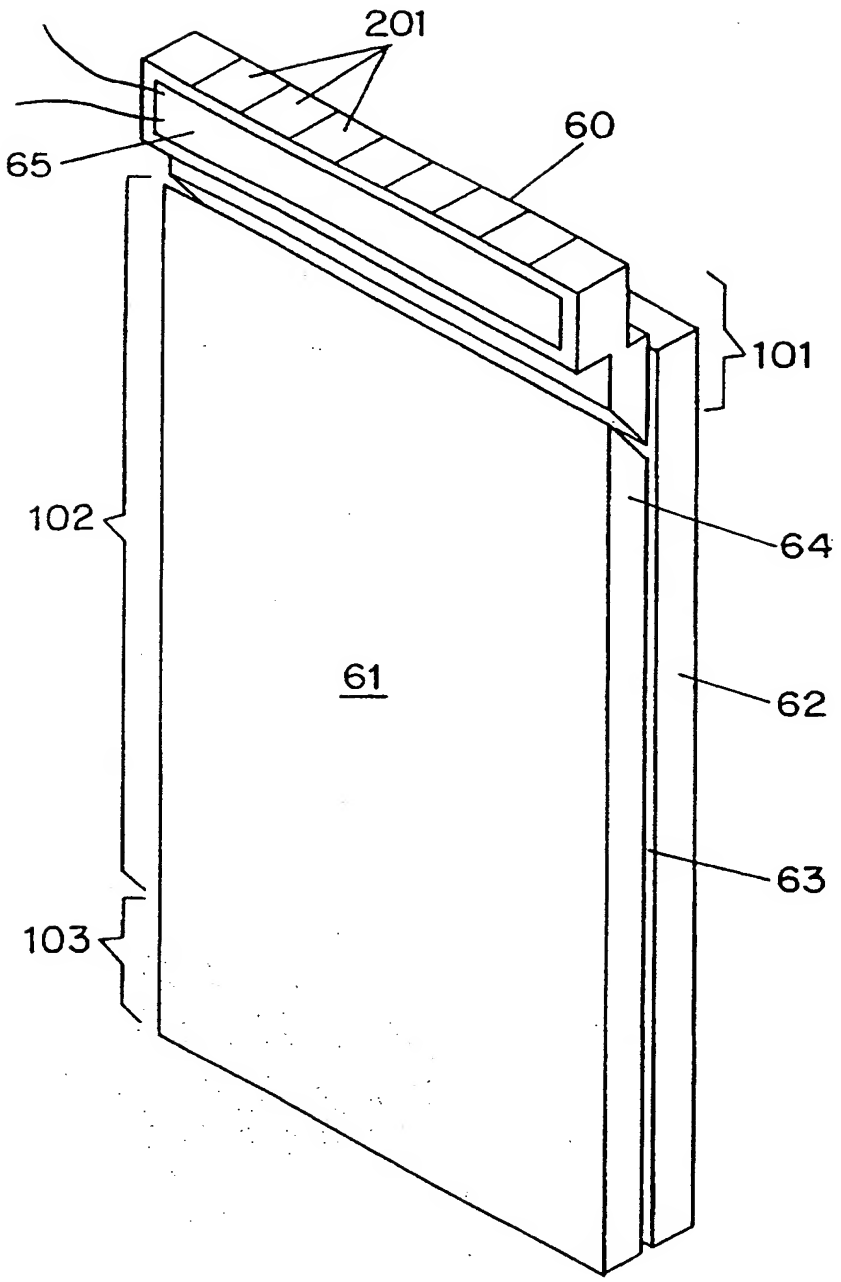

FIG. 6

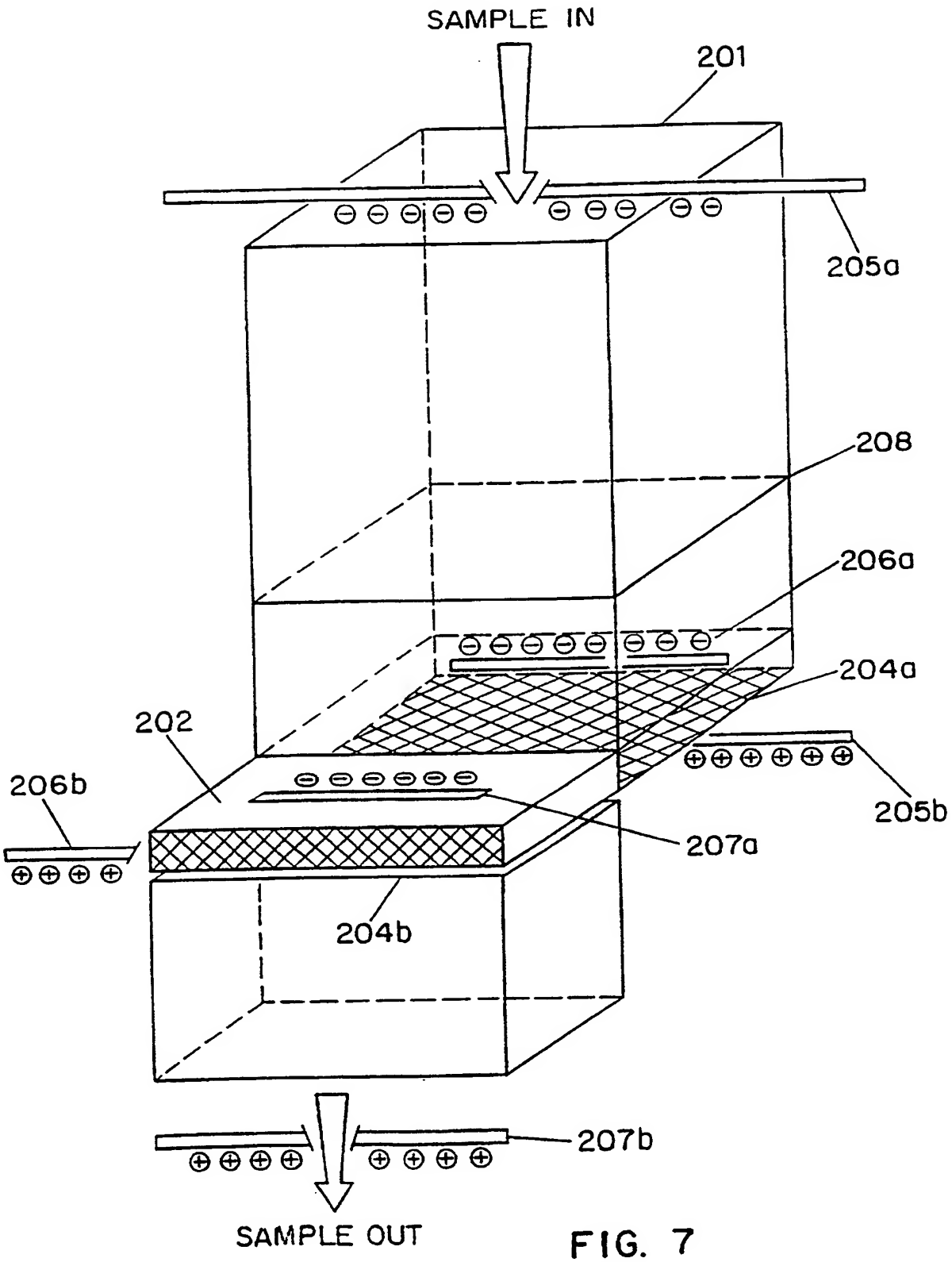

FIG. 7

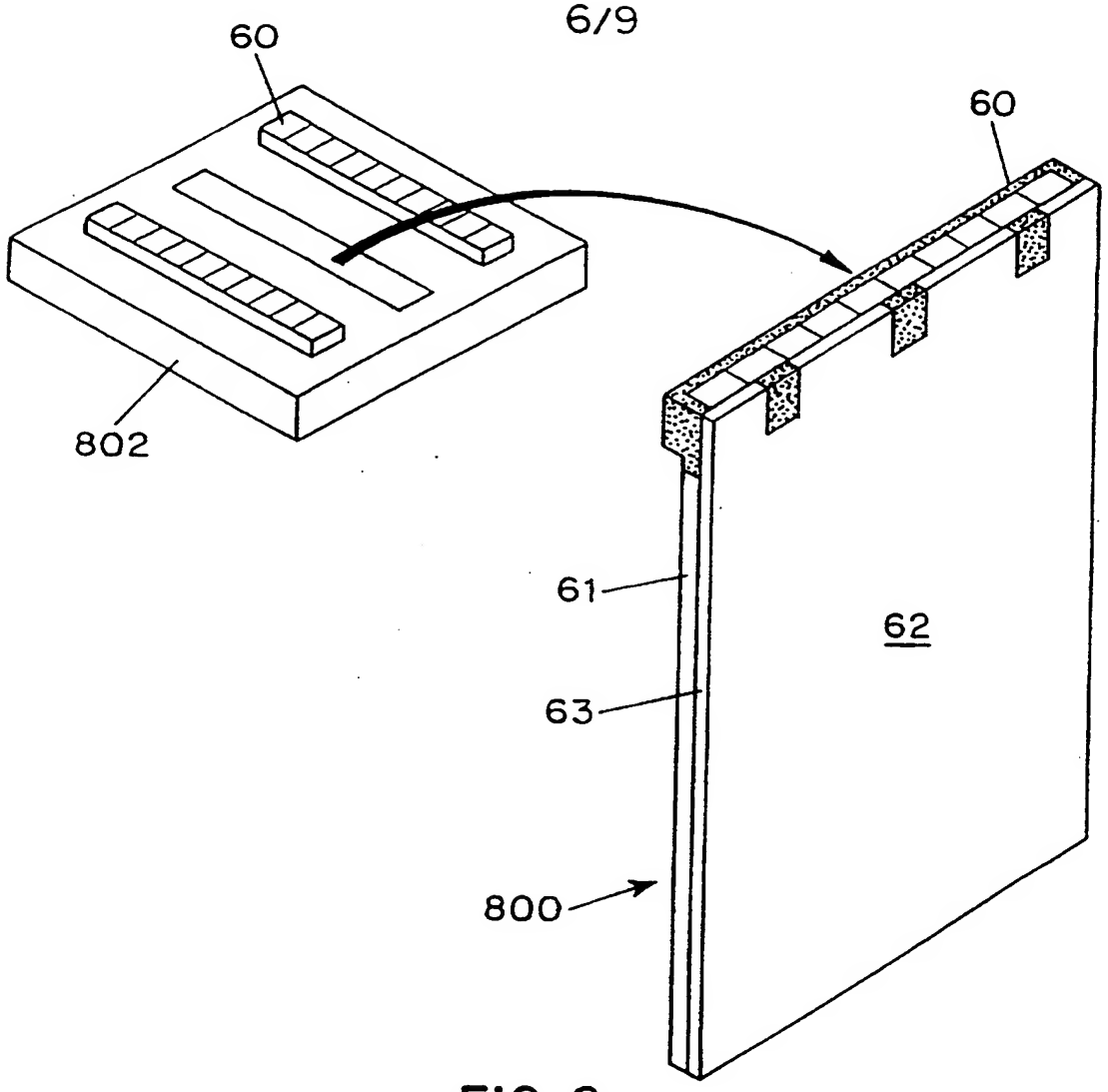

FIG. 8

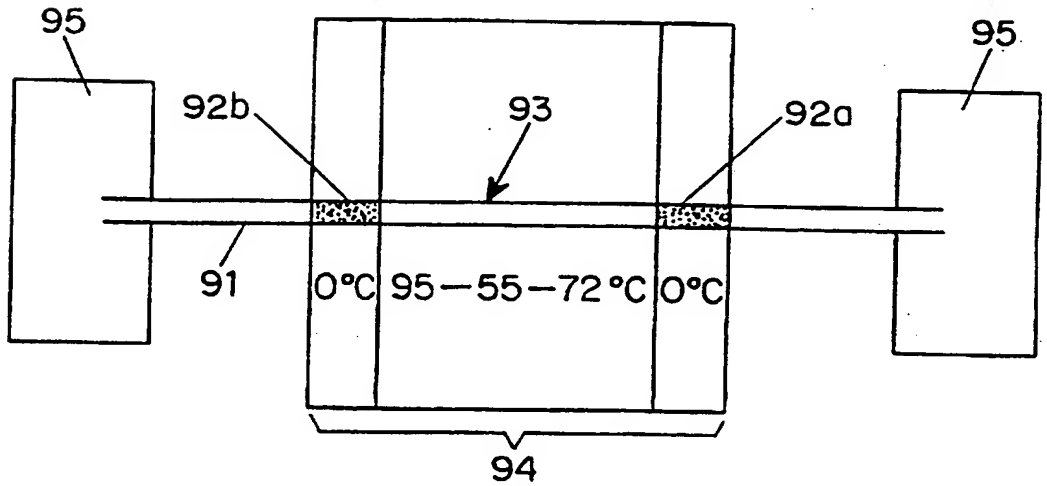

FIG. 9

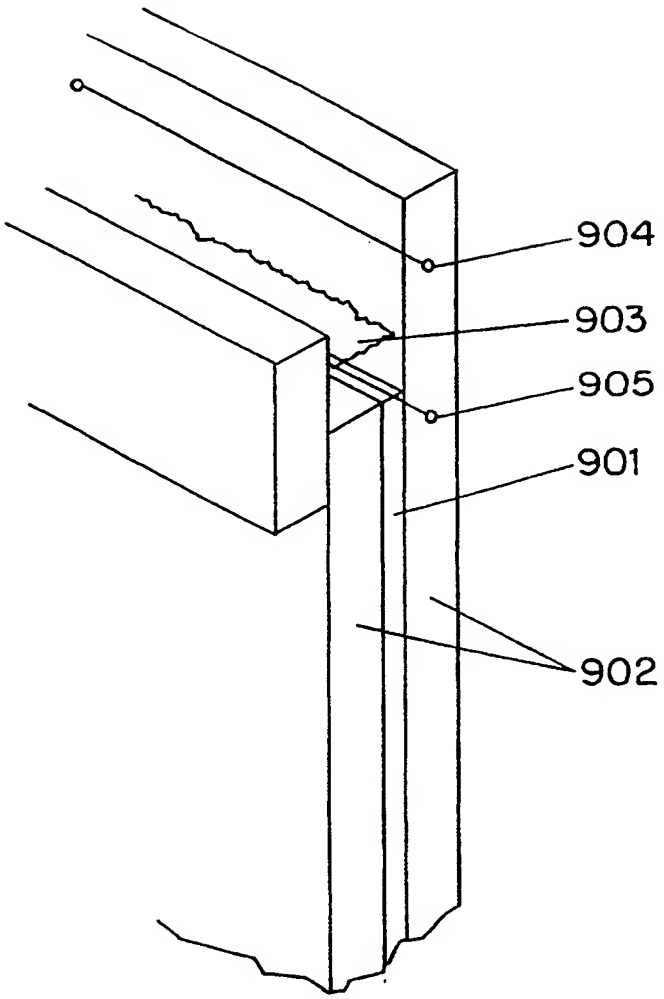

FIG. 10

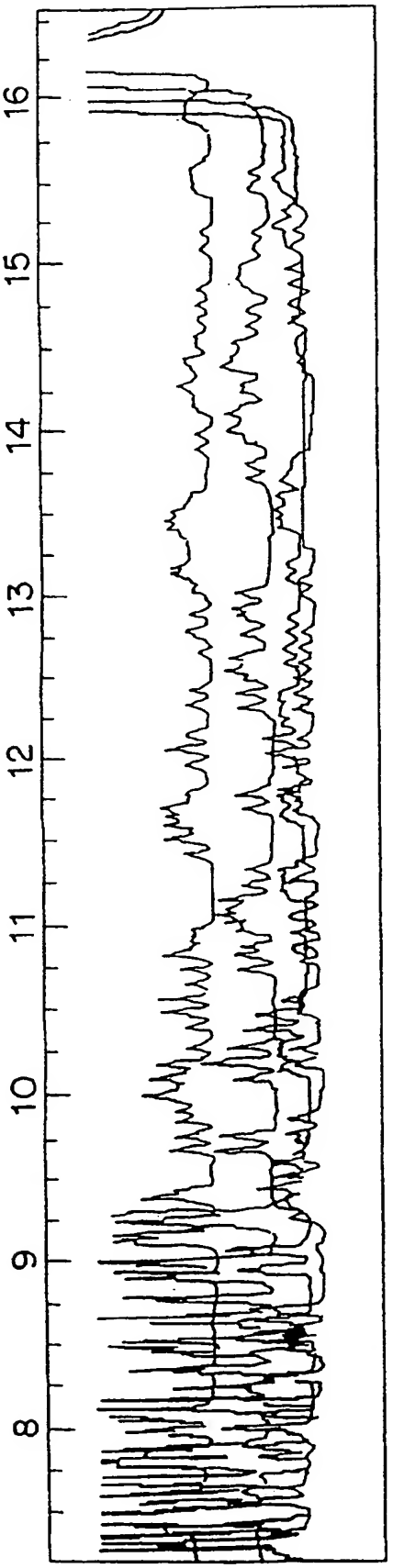

FIG. 11A

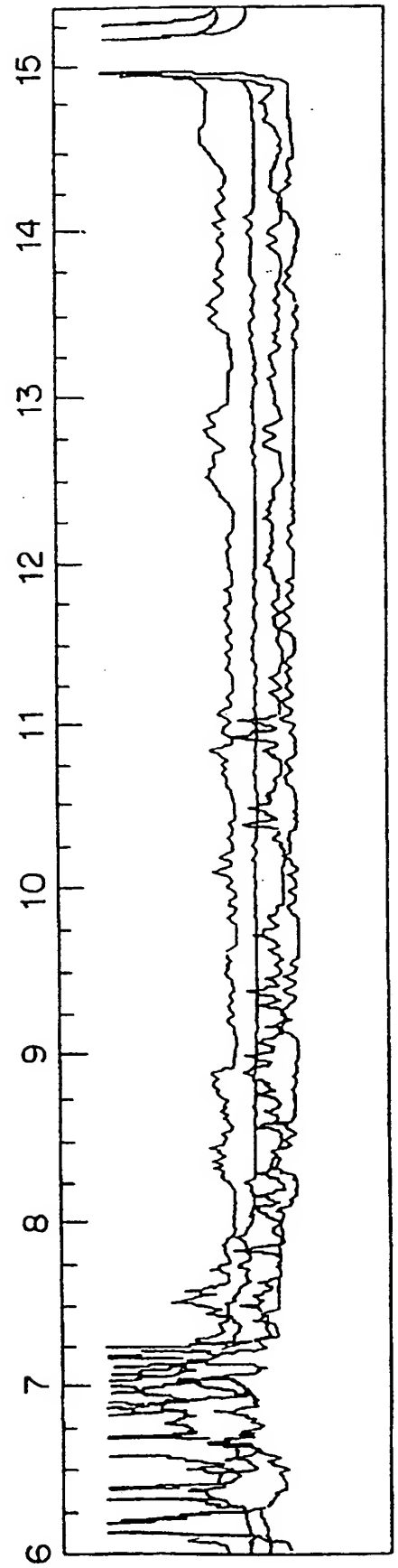

FIG. 11B

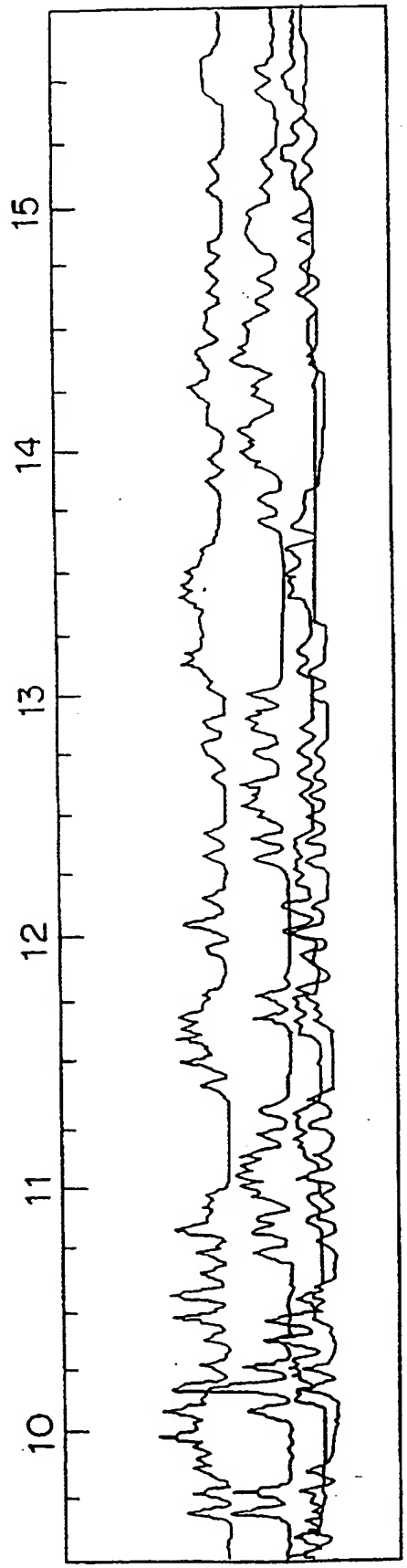

FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/15056

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N27/447 B01L7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 B01L G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 572 023 A (SHIMADZU CORP) 1 December 1993 see column 3, line 3 - column 5, line 8; figures	1,5,6, 10,11 2-4,7-9, 13-20
Y	WO 93 22058 A (UNIV PENNSYLVANIA) 11 November 1993 see page 20, last paragraph - page 21, paragraph 1	2,3
Y	see page 32, paragraph 2 - page 33, paragraph 1; figure 10	4
A	WO 96 18892 A (VISIBLE GENETICS INC ;WATERHOUSE PAUL (CA); RENFREW JOHN A (CA); S) 20 June 1996 see page 6, line 4 - line 30; figure 1	5,6
Y	see page 8, line 22 - page 9, line 8; figure 4	7-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 1997	15/01/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hocquet, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 08234 A (LABINTELLIGENCE INC) 14 April 1994 see page 4, line 20 - line 27; figure 1 see page 7, line 15 - line 36 see page 11, line 7 - line 29; figure 4	13-15, 17-20
Y	WO 93 02212 A (UNIV PARTNERSHIPS PTY LTD) 4 February 1993 see the whole document	16
P,X	WO 96 35810 A (CURAGEN CORP) 14 November 1996 see page 15, line 5 - line 28 see page 35, line 11 - page 36, line 8; figure 8 see page 39, line 1 - page 40, line 25	1,5, 17-19
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X	WO 91 07486 A (STAPLETON MARILYN J) 30 May 1991 see page 16, line 30 - line 35 see page 25, line 8 - line 36 see page 43, line 25 - page 46, line 25	1,5
E	WO 97 41259 A (VISIBLE GENETICS INC ;LEUSHNER JAMES (CA); HUI MAY (CA); DUNN JAME) 6 November 1997 see page 3, line 19 - page 4, line 10 see page 8, line 9 - page 9, line 20	16

INTERNATIONAL SEARCH REPORT

ormation on patent family members

Intern nal Application No
PCT/US 97/15056

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		DE 69303898 D	05-09-96
		DE 69303898 T	20-02-97
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